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PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

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United States Patent and Trademark Office
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in its capacity as elected Office

Date of mailing (day/month/year) 23 May 2000 (23.05.00)	
International application No. PCT/PT99/00015	**Applicant's or agent's file reference**
International filing date (day/month/year) 17 August 1999 (17.08.99)	**Priority date** (day/month/year) 21 August 1998 (21.08.98)
Applicant MEIRINHOS DA CRUZ, Maria, Eugénia et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

30 March 2000 (30.03.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [ ] was

[X] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

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Authorized officer

Juan Cruz

Telephone No.: (41-22) 338.83.38

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PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
010459		

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/PT99/00015	17/08/1999	21/08/1998

International Patent Classification (IPC) or national classification and IPC
A61K9/127

Applicant
INSTITUTO NACIONAL DE ENGENHARIA E TECNOLOGIA INDU

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
30/03/2000	14.11.2000

Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Lindner, A Telephone No. +49 89 2399 8640

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/PT99/00015

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-34	as originally filed	

Claims, No.:

1-18	with telefax of	26/10/2000

Drawings, sheets:

1/5-5/5	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 1-18
	No:	Claims
Inventive step (IS)	Yes:	Claims 2
	No:	Claims 1, 3-18
Industrial applicability (IA)	Yes:	Claims 1-17
	No:	Claims

2. Citations and explanations
   see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:
   D1 = WO 95/31970
   D2 = WO 86/01102

2. D1 discloses liposomes comprising phosphatidylcholine and trifluralin as active agent (D1: claims 1-5). D1 does not specifically relate to a mixture comprising liposomes above as well as below 100 nm. As a consequence, the subject-matter as claimed in claims 1 and 2 is novel (article 33(2) PCT).

3. According to p. 8 of the present application te beneficial effect in relation with parenteral administration of the liposome composition can only be obtained in the presence of two distinct populations (p. 8, l. 1-2). Claim 1 does not contain this feature, it relates to one population only with particle sizes both above and below 100 nm. In view of the fact that the technical problems obviously not solved by the subject-matter as claimed in claim 1, the requirements of article 33(3) PCT are not met. The same objections applies, mutatis mutandis to the subject-matter as claimed in present claims 17 and 18.

4. No objections are raised with regard to claim 2 in combination with claim 1. There, two distinct populations of liposomes are clearly claimed.

5. The subject-matter of claim 3 is new over D1, as D1 does not specifically relate to lyophilization and subsequent rehydration (article 33(2) PCT). However, lyophilization and rehydration is well-known in the art (D2: p. 14, l. 13-15; claims). It is additionally noted that addition of trehalose is also well known in the art so that the method as claimed in claims 3-16 does not involve an inventive step (article 33(3) PCT). The fact that in the prior art trehalose was used as cryoprotectant rather than as antisublimating agent is of no importance as the particular use of trehalose does not change the process step (adding trehalose). It is additionally noted that liposomes obtainable by the process as claimed in present claim 7 are already known (cf. novelty objection of present claim 1 above).

6. For the assessment of the present claim 18 on the question whether it is industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VIII

Certain observations on the international application

7. The back reference in claim 18 (1 to 6 and 21) is not correct.

Claims

1. A liposomal formulation characterized by the fact of containing one dinitroaniline incorporated or encapsulated, for example, trifluralin, for use in the preparation of a pharmaceutical formulation, characterized by the fact of containing a mixture of liposome populations with diameters respectively bigger and lower than 100 nm.

2. A liposomal formulation, according to claim 1, characterized by the fact that of mixing populations of particles, respectively bigger than 400 nm and lower than 100 nm.

3. Process for the preparation of a liposomal formulation containing one dinitroaniline, characterized by:

- obtention of a liposomal preparation containing a dinitroaniline by hydration, with a solution containing an antisublimating agents, for example trehalose, of a lipidic film containing the dinitroaniline .
- lyophilization of the dinitroaniline liposomal formulation
- rehydration of the dehydrated liposomal formulation

4. Process according to claim 3, characterized by the performing of the sizing step of the dinitroaniline liposomal formulation in order to reduce the vesicles diameter, done previously to the dehydration step.

5. Process, according to claim 4, characterized by the performing the sizing step by extrusion under pressure through porous membranes.

6. Process, according to any of the claims 3 to 5, characterized by the fact that the hydration is carried out by the addition of a small amount of an aqueous solution, followed by the addiction of the remaining volume of the aqueous solution, after a resting period.

7. Process, according to claim 6, characterized by the fact of using, in the hydration steps, a non-saline solution.

8. Process according to claim 7, characterized by the fact of performing the rehydration steps with saccharose, trehalose, glucose or any other sugar solution.

9. Process, according to any of the claims 3 to 8, characterized by the fact of mixing different diameter particle populations.

10. Process, according to claim 9, characterized by the fact of mixing particles that, after sizing, present population with diameters of, respectively, bigger and lower than 100 nm.

11. Process, according to claim 10, characterized by the fact of performing the sizing step according to claim 5.

12. Process, according to any of the claims 3 to 5 or 9 to 11, characterized by the fact that the hydration is performed according to claim 6.

13. Process, according to any of the claims 3 to 6 or 9 to 12, characterized by the fact of using in the hydration step a solution according to claim 7.

14. Process, according to any of the claims 3 to 7 or 9 to 13, characterized by the fact of using solutions according to claim 8.

15. Process, according to any of he claims 3 to 14, characterized by the use of any of the following lipids, hydrogenated or not, individually or in mixtures, in any molar ratio: distearoylphosphatidylcholine (DSPC), phosphatidylcholine (PC), cholesterol (Chol) or derivatives, sphingomielin (SM), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), phosphatidylglycerol (PG), dimiristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), gangliosides, ceramides, phosphatidylinositol (PI), phosphatydic acid, (PA),

dicetylphosphate (DcP), dimiristoylphosphatidylglycerol (DMPG), stearylamine (SA) dipalmitoylphosphatidylglycerol (DPPG) and other synthethic lipids.

16. Process, according to any of the claims 3 to 15, characterized by the fact of the dinitroaniline is trifluralin.

17. Liposomal formulations according to any of the claims 1 and 2, when prepared by a process according to any of the claims 3 to 16.

18. Use of the liposomal formulations for the preparation of a pharmaceutical formulation for the treatment in humans or animals, characterized by the use of a therapeutic quantity of a dinitroaniline liposomal formulation according to any of the claims 1 to 6 and 21.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/PT 99/00015

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K9/127 A61K31/136 A61P33/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 95 31970 A (AGRI TEK INC) 30 November 1995 (1995-11-30) page 10, line 23 -page 11, line 28 claims 1-5	1,2,6 1-3,6

Y	GENNARO AR, ET AL (EDS): "Remington's Pharmaceutical Sciences, 17th ed." 1985 , MACK PUBLISHING CO., EASTON, USA , ISBN: 0-912734-03-5 XP002127988 page 1644 -page 1661 & LONGER MA AND ROBINSON JR: "Sustained-release drug delivery systems" passage "Liposomes" page 1659 -page 1660	1-3,6

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 January 2000	03/02/2000
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Authorized officer Epskamp, S

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. ational Application No
PCT/PT 99/00015

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	CHAN MM, ET AL.: "Herbicides to curb human parasitic infections: In vitro and in vivo effects of trifluralin on the trypanosomatid protozoans" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 90, no. 12, 1993, pages 5657-5661, XP002127876 ISSN: 0027-8424 cited in the application page 5658, left-hand column, line 34 - line 51	1-22

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/PT 99/00015

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 22 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int...ational Application No
PCT/PT 99/00015

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9531970 A	30-11-1995	AU 701022 B	21-01-1999
		AU 2648095 A	18-12-1995
		CA 2190852 A	30-11-1995
		GB 2303791 A,B	05-03-1997
		US 5958463 A	28-09-1999

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
A61K 9/127, 31/136, A61P 33/02

A1

(11) International Publication Number: WO 00/10532

(43) International Publication Date: 2 March 2000 (02.03.00)

(21) International Application Number: PCT/PT99/00015

(22) International Filing Date: 17 August 1999 (17.08.99)

(30) Priority Data:
102197 21 August 1998 (21.08.98) PT

(71) Applicant *(for all designated States except US)*: INSTITUTO NACIONAL DE ENGENHARIA E TECNOLOGIA INDUSTRIAL/INSTITUTO DE BIOTECNOLOGIA, QUÍMICA FINA E TECNOLOGIAS ALIMENTARES [PT/PT]; Azinhaga dos Lameiros, Paço do Lumiar, P–1699 Lisboa Codex (PT).

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(75) Inventors/Applicants *(for US only)*: MEIRINHOS DA CRUZ, Maria, Eugénia [PT/PT]; Rua Maestro Raúl Ferrão, 43, P–1500 Lisboa (PT). CARVALHEIRO, Manuela, Colla [PT/PT]; Avenida Almirante Reis, 62–G, 4°, P–1150 Lisboa (PT). JORGE, João, Carlos, Santana [PT/PT]; Rua Acúrsio Pereira, 8, R/C A, Olivais Sul, P–1800 Lisboa (PT).

(74) Agent: ARNAUT, José, Luís; Rua do Patrocínio, 94, P–1399–019 Lisboa (PT).

(81) Designated States: BR, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DINITROANILINE LIPOSOMAL FORMULATIONS AND PROCESSES FOR THEIR PREPARATION

(57) Abstract

The invention refers to liposomal formulations containing one or several dinitroanilines, varying the liposome size from 50 μm to 0.01 μm with encapsulation efficiencies typically bigger than 30 %. When administered to animals, the liposomal dinitroanilines do not present acute toxicity or significantly diminish the toxicity of the free formula and are effective against infections by protozoarian or other microorganisms. The present invention refers also to a process for the preparation of liposomal formulations that comprises the preparation of multilamellar liposomes containing the dinitroaniline, to submit them to dehydration, rehydration and, optionally, to a sizing step before the dehydration. The dehydration is carried out in the presence of cryoprotectants in order to avoid sublimation and consequent loss of the drug in this step. The present invention refers also to dinitroaniline liposomal formulations containing a mixture of particles that after sizing, present populations superior and inferior to 100 nm in diameter.

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Description

DINITROANILINE LIPOSOMAL FORMULATIONS AND PROCESSES FOR THEIR PREPARATION

Field of Invention

This invention relates to liposomal compositions containing one or more dinitroanilines, incorporated or encapsulated, to processes for their preparation and to the use of these liposomal formulations in the treatment of infections in humans or animals.

The referred liposomal formulations can contain as dinitroaniline, for example, preferably, trifluralin (TFL).

Besides dinitroanilines, the liposomal formulations of the invention contain also phospholipids, individually or in mixtures, hydrogenated or not, with or without cholesterol (Chol) and electrically charged molecules, lipidic or not, as, for example, phosphatidylinositol (PI), phosphatidylglycerol (PG), dioleoylphosphatidylglycerol (DOPG), stearylamine (SA).

Invention background

The diseases caused by intracellular parasites of the mononuclear phagocytic system (MPS) cells are among the most important diseases all over the world due to the number of cases annually reported. One of these diseases is leishmaniasis, caused by a haemoflagellate protozoan named, in general, *Leishmania sp.* This disease has an incidence of at least 12 million infections in humans and animals. The dog has a crucial role as reservoir of the protozoan, being one among the responsible by the maintenance of zoonose. The disease is propagated from reservoirs to humans by vectors (sandflies). Leishmaniasis represents an immense public health problem in the Middle East, Africa, India,

China, Central and South America, and other tropical and subtropical ar as throughout the world like the Mediterranean region including Portugal (33, 34).

Leishmaniasis, depending on the subspecies, can assume several forms of the disease: visceral, mucocutaneous and cutaneous. The visceral form of the disease is usually fatal if not treated. All forms may be linger and recurrent despite the treatment with pentavalent antimonial compounds, the recommended first choice drugs (20, 26, 33, 34).

Leishmania sp are able to live in the mononuclear phagocytic system cells, in an intracellular vesicle inside the host cell (2, 5, 14, 16, 31). The fusion of host cell lysosomes with the vacuole containing the parasite, does not prevent the *leishmania* multiplication. This fusion can even supply the necessary nutrients for its multiplication. In this way, the parasite seems to be safe inside the cell, being this one of the reasons why its elimination is so difficult. This fusion mechanism lysosome-vacuole can be used for alternative therapies namely through the internalisation mechanism of liposomes by MPS cells (2).

Several different classes of drugs have been used to treat leishmaniasis, namely pentavalent antimonial compounds, trivalent antimonial compounds, antibiotics (polyenics, aminoglucosides), immunomodulators (interferon α) and chelating agents (desferrioxiamine), among several others (26, 30, 32, 35).

The present recommended treatment for canine leishmaniasis is a course of pentavalent antimonial drugs, either sodium stibogluconate or meglumine antimoniate. These drugs have a limited effectiveness and they do not achieve a complete cure of the disease. These therapies are accompanied by a combination of problems, particularly: variable efficacy, long course of treatments and severe side effects such as cardiac and renal toxicities. Increased resistance to treatment with pentavalent antimonial drugs has also been reported and attributed to inadequate treatments (9, 20, 24, 26, 32, 33, 35).

Other drugs, used in the treatment of leishmaniasis, have limited clinic application also due to severe side effects, such as amphotericin B that is nephrotoxic (14, 20) and methotrexate (MTX) with cardiac toxicity (10, 11).

In spite some progresses in the development of new drugs have been achieved, none has 100% success in the treatment of the disease. Besides the referred toxic effects, the small efficacy of treatments is the other main disadvantage of the used drugs against infections by *Leishmania sp* (14).

As examples of these drugs it can be referred methotrexate (85% reduction on the infection level) (10, 11) pentamidine (less efficacy than antimonial derivatives), dehydroemetine, achieving 67% of cure (1) and desferrioxiamine, with 44% efficiency (32). In the cases of treatment failure with pentavalent antimonials, subsequent treatments with other classes of drugs, such as pentamidine, amphotericin B, ketoconazole and paramomycin, do not significantly increase the results. Also formycin B, sinefungin and lepidine WR6026 showed high antileishmanial activity when compared to pentavalent antimonials, but toxicity problems persist (26, 27).

Allopurinol and related compounds (allopurinol nucleoside, thiopurinol, thiopurinol ribonucleoside) have been tested *in vitro* and *in vivo* (27). The protozoans are not able to synthesise purines, being dependent on host purines and nucleosides. The presence of inosine analogues (e.g. allopurinol ribonucleoside) inhibits the purine metabolising enzymes of the parasite, affecting RNA function and reducing protein synthesis (26, 27). Previous studies showed that allopurinol, allopurinol nucleoside and thiopurinol ribonucleoside have small activity in animal models of the disease, probably due to the small residence time and low serum levels obtained by these drugs (27).

Antibiotics, such as, streptomycin and trobamycin inhibit the growth of both promastigote and amastigote forms of the parasite (25).

Trifluralin is a herbicide known to be active against leishmaniasis. This drug binds to plant tubulins but not to animal tubulins. *Leishmania sp* tubulins are very similar to plant tubulins. In this way, trifluralin showed to be able to inhibit promastigote proliferation, to reduce promastigote to amastigote transformation, to interfere with amastigote replication and to reduce amastigote infectivity. *In vitro*

studies confirmed efficiency against all forms of leishmaniasis, but *in vivo* studies only presented good results against the cutaneous forms of the disease. A drug delivery system need to be developed for the use of trifluralin against visceral forms since trifluralin solubility and lipophilicity do not allow the administration by any other route than the topical one. By this route no activity against the visceral forms was observed (12).

In view of the difficulties above described, an alternative approach to the search of new drugs is the drug encapsulation in macrophage directed carriers, as liposomes.

Liposomes are phospholipid synthetic bilayer vesicles able of incorporating a variety of substances independently of their molecular weight, electrical charge and solubility (18, 19, 23).

The rationale for the use of liposomal associated drugs instead of free drugs for the treatment of visceral leishmaniasis rely on the fact that amastigotes of the parasite are specifically located in liver spleen and bone marrow macrophages. As liposomes are preferentially taken up by these cells (2, 5, 30), they can deliver toxic agents straight to the intracellular location of established parasites (28). Though, the administration of liposome-encapsulated agents theoretically increases the therapeutic index of the agent in two ways: 1) increasing the uptake of the carrier and consequently of the drug by macrophages contained organisms, and 2) reducing toxicity of the free drug due to relatively low uptake of carrier by organs to which the drug is toxic (2, 6, 16, 20).

The great majority of drug delivery systems administered by intravenous route are taken up from circulation by the liver, meaning that they accumulate preferentially in this organ, not achieving, at significant quantities, other organs also belonging to MPS (spleen and bone marrow). The uptake by these other organs can be increased by the reduction on vesicle diameter (8). This can result in the suppression of the infection in the spleen and bone marrow, quite difficult to achieve with the free drugs.

Results in lit rature show that liposome encapsulated drugs are much safer and more effective to treat MPS infection as compared to free drugs (5, 17, 28).

Liposomal formulations of pentavalent antimonials can increase 200 to 700 times the therapeutic index compared with the free form, depending upon the lipid composition of liposomes (4, 7, 8, 13). Liposomal amphotericin B is 2 to 5 times more active than free form (6, 29). Liposomal primaquine presents activity at doses not actives for the free form (3). However these results are strongly dependent from the infection stage at the beginning of treatment and are difficult to correlate due to the heterogeneity of the experimental conditions (15).

Most of the above described results suffer from limitations of the kind of liposomes used, with low encapsulation efficiency and of small half lives, not reaching crucial targets for the treatment of this disease and, also, because of high costs. Liposomal amphotericin B was effective in curing a few cases of visceral leishmaniasis in humans (16, 21, 22), but the cost of such a treatment is too high for widely application to animals infected population.

Invention Detailed Description

The present invention refers to liposomal formulations containing one or several dinitroanilines e to processes for their preparation.

The present invention concerns the achievement, under stable form, lyophilised or not, of liposomal formulations containing one or several dinitroanilines, for example trifluralin incorporated or encapsulated.

In the formulations obtained according to the present invention, the liposomal diameter varies between 0,01 µm to 50 µm. According to one of the preferred forms of preparation, mixtures of different size populations exist in the formulations of the present invention, with diameters respectively bigger and lower than 100 nm in a specially preferred form.

Additionally the present invention formulations may contain any of the following lipids, hydrogenated or not, individually or in mixtures, in any molar ratio: distearoylphosphatidylcholine (DSPC), phosphatidylcholine (PC), cholesterol (Chol) or derivatives, sphingomielin (SM), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), phosphatidylglycerol (PG), dimiristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC),

gangliosides, ceramides, phosphatidylinositol (PI), phosphatydic acid (PA), dicetylphosphate (DcP), dimiristoylphosphatidylglycerol, (DMPG), stearylamine (SA), dipalmitoylphosphatidylglycerol (DPPG) and other synthetic lipids.

The preparation process of the present invention liposomal formulations comprises the steps of:

- hydration from a lipid film containing the dinitroaniline for the achievement of a liposomal formulation
- lyophilization
- rehydration

In a common way, solubilization in organic solvent of the lipidic components and the dinitroaniline or dinitroanilines, for example trifluralin, can be done, followed by drying under N_2 stream or under vacuum, for example, in a rotavapor with controlled temperature for the achievement of a mixed homogeneous film of lipid and dinitroaniline or dinitroanilines, for example, trifluralin. This film can be, subsequently, hydrated with a sugar solution forming multillamelar liposomes. The following step can be the liposomal formulation sizing, under pressure, by successive extrusions through polycarbonate membranes of pore sizes varying from 5,0 to 0,01 µm. The sizing will end preferably after extrusion through the membrane with the desired pore size for a part of the population. After the attainment of the necessary different populations with well-determined diameter, the following step is the mixture of these populations.

After the attainment of the necessary different populations of well-determined diameter, the following step is the mixture of these populations. After the mixture of the populations, it can be, or not, done a concentrative dialysis, using, for example, polyethyleneglycol as hygroscopic agent, followed by a step of dehydration. This dehydration occurs preferably in the presence of sugars that will act as protective of sublimation of the dinitroaniline or dinitroanilines, for example, trifluralin.

The formulations according the present invention so obtained, after hydration with water, are ready for use.

Up to now, there is no literature reference to liposomal preparations with dinitroanilines.

According the present invention, in order to prepare the multillamelar liposomes a step of drying a mixture of one dinitroaniline, namely trifluralin, and lipids, both solubilized in the same solvent or mixture of organic solvents, is performed. The amount of trifluralin varies according the final volume to prepare, ranging from 10 µg to 1 g or more. The amount of lipid also changes according the final volume to be prepared, ranging from 1 µmole to 1 mole or more. The adequate lipids, hydrogenated or not for the preparation of the formulations are present individually or in mixtures, in any molar ratio from the following lipids: distearoylphosphatidylcholine (DSPC), phosphatidylcholine (PC), cholesterol (Chol) or derivatives, sphingomielin (SM), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), phosphatidylglycerol (PG), dimiristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), gangliosides, ceramides, phosphatidylinositol (PI), phosphatydic acid (PA), dicetylphosphate (DcP), dimiristoylphosphatidylglycerol, (DMPG), stearylamine (SA), dipalmitoylphosphatidylglycerol (DPPG) and other synthetic lipids.

The so obtained mixture is submitted to a step of drying under a N_2 stream, until the total remove of the solvent or mixture of solvents. After drying, hydration of the mixture with a solution of a sugar as, for example, trehalose, is done, ranging its concentration from 0,01 M to 2 M, under mechanical stirring or manual external stirring. The so obtained liposomal formulation is, then, submitted to a step of sizing, for example, by successive passages under pressure through polycarbonate filters of decreasing pore diameter, normally referred as extrusion. Extrusion starts normally through 5 µm diameter pore membranes and continues with passages through diameter pore membranes of 2, 1, 0,8, 0,6, 0,4, 0,2, 0,1 and 0,05 µm, reaching some times 0,02 µm. According to a preferred way preparation of the invention and after passage through 0,4 µm membranes, the so obtained liposomal preparation is split in two parts. Only one of those parts goes trough the rest of the extrusion procedure until, for example, 0,05 µm diameter

pore membranes. At the end, the two parts that correspond to two distinct populations are mixed, achieving, by this way, one liposomal formulation containing liposomes that exhibit two different diameter distribution populations. The simultaneous presence of these different diameter populations present the advantage that, after *in vivo* parenteral administration, the population of bigger diameter is rapidly captured by mononuclear phagocytic system cells, while the small size population remains in circulation, possibly reaching organs other than liver and spleen, where the parasitic infection also exist, as for example, the bone marrow.

The so obtained formulation may be, or not, submitted to a step of concentration by dialysis against, for example, polyethyleneglycol that will act as a water removing agent. After this dialysis step, the formulation can be frozen up to –70ºC during, at least one hour, after what it is submitted to lyophilization

After this lyophilization, the formulation is ready to be used, being enough for that, the addiction of water to the so obtained powder. Hydration occurs instantaneously originating one homogeneous suspension in water of liposomes, containing the dinitroaniline or dinitroanilines as, for example, trifluralin.

A particularly preferred way of preparation of the present invention is the one in which to a lipid mixture of DOPC:DOPG in a molar ratio of 7 : 3, in a total of 10 µmole of lipid, solubilized in chloroform, is added 1 µmole of trifluralin, solubilized in chloroform. The obtained mixture is, then, dried under a stream of nitrogen until total evaporation of the chloroform. The so film is hydrated with 0,1 mL of 0,3 M trehalose, with manual stirring. After complete resuspension of the lipidic film, the formulation rests for 15 minutes, after what 0,1 mL more of the same trehalose solution is added. Another 15 minutes resting period is allowed after what hydration is completed by adding 0,8 mL of the same solution. The so obtained liposomal formulation is submitted to a sizing step by passage under pressure through polycarbonate membranes of successively decreasing pore diameters, from 5 µm to 0,4 µm. After this extrusion procedure, the formulation is

divided in two equal parts. One of those parts continues the sizing step until a filter of 0,05 µm. The two populations so obtained are, then, mixed. The mixed liposomal formulation is submitted to freezing at –70ºC for 60 minutes and, after that period, lyophilised. In this way, a liposomal formulation ready to be hydrated with 1,0 mL of distilled sterile water is obtained, able to be parenterically administered.

The formulations may also contain auxiliary substances, pharmaceutically acceptable, useful for preservation of their quality and or to turn them closely related to physiological conditions, such as pH adjusting agents, buffering agents, tonicity agents, antioxidants and other adjuvants as, for example, sodium acetate, sodium lactate, sodium chloride, potassium, chloride, calcium chloride, glucose, saccharose, mannitol, xylitol, alpha-tocopherol.

The pharmaceutical formulations obtained according the present invention can be administered to warm blood animals, such as man, already suffering from leishmaniasis, during the necessary time interval and in a necessary quantity to end or significantly inhibit infection progress. The adequate quantities for the achievement of that effect are named as "therapeutically efficient doses". The therapeutic efficient doses for this use will depend on the infection degree and on the general state of health of the treatment individual. There is no other formulation of the free drug, namely, trifluralin, used in parenteric administration.

The following examples, of liposomal formulations prepared according the present invention and of their respective physico-chemical and biological analysis, are presented as illustrations and not as limitations.

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Examples

These examples illustrate liposomal formulation prepared according to the present invention and processes for their preparation in which the used dinitroaniline is trifluralin.

Trifluralin (TFL) incorporation in liposomes

The preparation of the following described formulations, in a total volume of 5 mL for lipidic composition, started by the addition of TFL to lipid in chloroform, followed by evaporation of the solvent under nitrogen stream. Hydration of the resulting film was done by adding 500 µL of trehalose 0,3 M, stirring and resting for 15 minutes, addition of 500 µL more of 0,3 M trehalose, stirring again and resting again for a new 15 minute period and, finally, by the addition of 4000 µL of 0,3 M trehalose. Samples for dosage (initial TFL and initial lipid) were removed. The liposomal formulations so obtained were sized by successive filtration, under nitrogen pressure, through polycarbonate filters with pores of 5,0, 2,0, 1,0, 0,8, 0,6 and 0,4 µm, with two passages in the last filter (extrusion). The non-incorporated TFL, as it is insoluble in aqueous solutions, crystallises on a needle type structure and remains at the top of the filters. After extrusion through 0,4 µm filter, the formulations are split in two equal parts. With one of those part extrusion procedure continues, now through diameter pore membranes of 0,2 and 0,1 µm, with two passages in the last filter. The liposomal formulation half part that was extruded until membranes of 0,4 µm pore diameter, is named VET400 (Vesicles Extruded Through 400 nm). The liposomal formulation half part that was extruded until membranes of 0,1 µm pore diameter, is named VET400 (Vesicles Extruded Through 100 nm). The VET400 and VET100 formulations obtained by the previous process are finally submitted to dosage (final TFL and final LIP).

Table 1a represents the lipid composition effect on the incorporation parameters of liposomes sized until 0,4 µm pore filters. **Table 1b** represents the lipid

composition effect on the incorporation parameters of liposomes sized until 0,1 µm pore filters. The formulations were prepared with different lipid compositions, with lipid and TFL quantities presented in the referred tables. Incorporation efficiency (I.E.) represent the ratio between the final (drug to lipid ratio) and the initial (drug to lipid ratio) and is expressed as a percentage. The recovery is also expressed as a percentage and can be referred to drug (TFLf/TFLi) or to lipid (LIPf/LIPi).

TABLE 1a - Lipid composition effect on incorporation parameters of TFL in liposomes sized until 0.4 µm

Formulation number	Lipidic composition Molar Ratio		TFLi (µg)	TFLf (µg)	LIPi (µmol)	LIPf (µmol)	(TFL/LIP)i (g/mol)	(TFL/LIP)f (g/mol)	LIPf/LIPi (%)	TFLf/TFLi (%)	I.E. (%)
1	PC:CHOL	average	332,03	140,78	11,52	9,48	29,09	14,37	81,72	44,89	52,97
	2:1	standard deviation	43,50	75,14	0,82	2,24	5,80	5,65	13,62	28,53	27,54
		median	330,73	144,47	11,41	8,25	28,99	17,52	75,55	43,68	60,43
2	DMPC:CHOL	average	367,60	110,90	12,58	10,20	30,02	11,30	80,37	30,31	37,95
	2:1	standard deviation	50,82	14,34	3,02	3,00	5,71	2,16	6,08	3,55	6,17
		median	346,53	119,18	13,87	10,75	28,85	11,08	77,52	28,53	37,42
3	DSPC:CHOL	average	390,66	170,81	13,48	10,75	29,13	15,88	79,86	44,09	55,24
	2:1	standard deviation	64,63	29,75	1,33	1,03	5,04	2,37	4,10	8,05	9,76
		median	358,40	185,56	13,76	10,35	29,79	15,56	81,42	40,92	54,40
4	DPPC:CHOL	average	322,83	84,72	9,14	7,38	35,37	11,17	80,88	24,75	31,59
	2:1	standard deviation	31,36	82,60	0,98	0,87	2,14	10,17	6,80	23,86	29,64
		median	334,68	89,15	8,85	7,31	34,43	13,61	80,99	26,64	35,99
5	HPC:CHOL	average	328,77	128,65	10,34	8,13	36,97	16,55	81,90	39,41	49,77
	2:1	standard deviation	24,77	11,07	3,92	2,32	20,48	3,77	14,78	5,79	14,16
		median	330,73	133,40	12,46	8,45	26,54	16,17	80,69	41,29	54,55
6	PC:CHOL	average	309,65	190,30	10,40	8,49	29,78	22,42	81,61	61,42	75,26
	4:1	standard deviation	8,22	11,06	0,23	0,10	1,37	1,12	2,40	2,06	0,62
		median	307,02	190,30	10,30	8,45	29,81	22,63	81,65	61,98	75,19
7	HPC:CHOL	average	334,68	114,96	11,87	8,49	29,71	13,29	71,63	35,35	50,85
	4:1	standard deviation	47,42	52,36	2,77	2,46	10,81	3,61	11,53	16,98	25,27
		median	334,68	109,70	11,95	7,16	24,03	14,99	77,52	38,19	65,43
8	PC:PG	average	318,87	323,03	11,85	11,90	26,90	27,15	100,40	101,34	100,93
	4:1	standard deviation	3,95	8,51	0,16	0,12	0,19	0,99	0,43	3,85	3,59
		median	318,90	326,70	11,76	11,86	26,81	27,55	100,34	102,45	101,58
9	DMPC:CHOL	average	388,04	117,60	12,19	9,56	31,98	12,37	78,46	30,27	38,63
	4:1	standard deviation	69,86	22,30	2,40	1,87	2,42	1,24	4,06	0,70	1,59
		median	370,24	114,44	12,60	10,46	32,37	13,06	77,03	30,38	38,32
10	DSPC:CHOL	average	325,36	135,52	11,76	9,19	28,87	15,22	79,99	42,08	52,47
	4:1	standard deviation	41,59	12,68	3,52	1,78	6,17	3,72	9,53	6,33	1,76
		median	336,29	136,60	11,99	9,29	30,06	15,89	77,48	40,96	52,87

The presented values are the average, standard deviation and median of, at least, three preparations

Abbreviations:

TFLi Inicial trifluralin
TFLf Final trifluralin
LIPi Initial lipid
LIPf Final lipid
(TFL/LIP)i Initial ratio TFL/LIP
(TFL/LIP)f Initial ratio TFL/LIP
LIPf/LIPi LIP recovery
TFLf/TFLi TFL recovery
I.E. Incorporation efficiency

TABLE 1a - Lipid composition effect on incorporation parameters of TFL in liposomes sized until 0.4 µm (cont.)

Formulation number	Lipidic composition Molar Ratio		TFLi (µg)	TFLf (µg)	LIPi (µmol)	LIPf (µmol)	(TFL/LIP)i (g/mol)	(TFL/LIP)f (g/mol)	LIPf/LIPi (%)	TFLf/TFLi (%)	I.E. (%)
11	DPPC:CHOL	average	318,22	82,83	9,11	7,82	34,72	10,26	86,15	24,39	28,97
	4:1	standard deviation	64,51	55,90	1,22	0,79	2,47	6,43	5,17	14,06	17,36
		median	310,97	97,05	9,18	7,52	33,86	12,90	84,64	31,21	38,10
12	PC:CHOL (Soy bean)	average	397,89	165,01	10,45	7,68	39,69	21,68	73,84	41,25	56,09
	4:1	standard deviation	22,78	32,43	2,63	1,83	9,65	1,37	5,23	5,84	9,00
		median	395,90	166,59	11,93	8,09	35,10	21,23	76,62	42,08	60,49
13	DPPC:DPPG	average	301,09	101,27	9,49	6,12	31,76	16,70	64,29	33,63	52,49
	7:3	standard deviation	13,69	7,80	0,50	0,98	1,80	1,69	7,18	2,16	2,47
		median	293,19	104,95	9,67	6,12	32,76	17,41	63,26	33,62	53,14
14	DOPC:DOPG	average	323,50	178,09	10,35	8,17	31,31	22,04	78,27	55,36	71,36
	7:3	standard deviation	37,57	33,67	0,80	2,05	3,40	2,14	13,64	10,93	14,41
		median	324,80	185,56	10,37	7,67	32,35	22,02	73,99	57,55	64,66
15	PC:CHOL:PG	average	313,63	148,11	12,33	10,44	25,69	13,86	85,09	46,77	55,25
	10:5:1	standard deviation	44,63	58,94	2,19	1,50	3,48	3,71	5,56	14,64	18,66
		median	309,00	139,70	11,82	10,75	24,48	13,00	84,43	51,45	56,58
16	PC:CHOL:PI	average	337,98	143,42	12,23	9,45	27,98	15,17	76,72	41,89	54,68
	10:5:1	standard deviation	40,38	42,01	2,55	2,67	2,73	0,72	8,44	7,00	7,22
		median	320,85	120,76	11,13	8,40	27,76	15,38	80,69	38,06	56,78
17	PC:CHOL:SA	average	290,55	95,05	10,54	8,32	27,79	10,72	77,60	31,24	38,37
	10:5:1	standard deviation	35,45	81,33	1,89	2,85	2,09	6,87	12,71	23,70	25,01
		median	277,39	81,25	10,38	7,39	26,72	13,42	71,16	30,83	44,43
18	DPPC:CHOL:DPPG	average	324,80	23,82	9,03	6,93	35,87	3,36	76,96	7,53	9,42
	10:5:1	standard deviation	47,95	10,52	0,26	1,26	4,34	1,24	16,02	3,71	3,38
		median	338,63	29,09	9,15	6,82	37,01	3,53	73,96	8,42	11,37
19	PC:CHOL:DSPE-PEG	average	231,29	213,26	9,47	9,01	24,41	23,69	95,12	92,39	97,10
	3.7:1:0.3	standard deviation	13,16	3,65	0,06	0,28	1,26	0,97	3,51	5,15	2,95
		median	227,99	215,37	9,46	9,12	24,20	23,36	96,82	91,69	96,20
20	DSPC:CHOL:DSPE-PEG	average	322,62	192,21	10,20	8,26	32,73	22,96	80,10	64,79	76,58
	8:2:0.5	standard deviation	60,28	85,16	1,68	2,61	10,23	5,35	14,25	42,28	36,40
		median	350,48	171,01	10,45	7,11	34,86	25,42	76,43	48,79	63,84

The presented values are the average, standard deviation and median of, at least, three preparations

Abbreviations:

TFLi Inicial trifluralin *LIPi* Initial lipid *(TFL/LIP)i* Initial ratio TFL/LIP *LIPf/LIPi* LIP recovery

TFLf Final trifluralin *LIPf* Final lipid *(TFL/LIP)f* Initial ratio TFL/LIP *TFLf/TFLi* TFL recovery

I.E. Incorporation efficiency

TABLE 1b - Lipid composition effect on incorporation parameters of TFL in liposomes sized until 0.1 µm

Formulation number	Lipidic composition Molar Ratio		TFLi (µg)	TFLf (µg)	LIPi (µmol)	LIPf (µmol)	(TFL/LIP)i (g/mol)	(TFL/LIP)f (g/mol)	LIPf/LIPi (%)	TFLf/TFLi (%)	I.E. (%)
1	PC:CHOL	average	332,03	70,67	11,52	7,15	29,09	9,47	61,60	22,54	34,74
	2:1	standard deviation	43,50	42,29	0,82	2,39	5,80	2,71	17,24	16,29	16,65
		median	330,73	46,79	11,41	6,68	28,99	9,29	62,05	14,15	32,06
2	DMPC:CHOL	average	367,60	48,20	12,58	8,02	30,02	6,56	64,07	13,32	20,74
	2:1	standard deviation	50,82	18,51	3,02	1,73	5,71	3,96	3,22	5,94	8,80
		median	346,53	49,95	13,87	8,90	28,85	5,61	65,81	11,74	19,45
3	DSPC:CHOL	average	390,66	94,90	13,48	8,18	29,13	12,09	61,43	24,80	43,97
	2:1	standard deviation	64,63	10,82	1,33	1,60	5,04	3,87	15,02	5,48	22,33
		median	358,40	92,10	13,76	8,45	29,79	10,15	69,97	23,93	34,07
4	DPPC:CHOL	average	322,83	65,26	9,14	6,46	35,37	8,35	69,94	18,93	24,09
	2:1	standard deviation	31,36	88,39	0,98	1,77	2,14	10,04	11,69	25,45	29,91
		median	334,68	29,93	8,85	5,49	34,43	5,55	65,79	8,94	14,69
5	HPC:CHOL	average	328,77	100,88	10,34	8,06	36,97	13,55	79,50	30,89	39,15
	2:1	standard deviation	24,77	6,17	3,92	2,64	20,48	4,81	6,53	4,26	7,08
		median	330,73	98,42	12,46	9,19	26,54	10,85	78,13	29,76	40,34
6	PC:CHOL	average	309,65	114,22	10,40	6,18	29,78	18,48	59,42	36,89	62,12
	4:1	standard deviation	8,22	3,80	0,23	0,07	1,37	0,40	1,43	1,11	2,89
		median	307,02	115,28	10,30	6,20	29,81	18,59	59,22	36,81	60,50
7	HPC:CHOL	average	334,68	73,83	11,87	7,42	29,71	9,72	62,43	22,60	36,47
	4:1	standard deviation	47,42	30,47	2,77	2,00	10,81	1,63	6,04	9,88	15,63
		median	334,68	67,86	11,95	6,63	24,03	10,23	65,45	23,62	42,58
8	PC:PG	average	318,87	324,60	11,85	12,45	26,90	26,08	105,06	101,82	96,95
	4:1	standard deviation	3,95	2,97	0,16	0,32	0,19	0,86	2,34	2,15	3,51
		median	318,90	325,90	11,76	12,63	26,81	25,80	105,07	102,20	95,16
9	DMPC:CHOL	average	388,04	52,41	12,19	7,22	31,98	6,96	57,76	13,11	21,95
	4:1	standard deviation	69,86	26,11	2,40	2,74	2,42	1,29	14,36	5,49	4,90
		median	370,24	63,65	12,60	8,75	32,37	7,19	60,88	15,27	24,45
10	DSPC:CHOL	average	335,36	87,53	11,76	7,98	29,53	11,01	69,57	25,93	37,51
	4:1	standard deviation	48,56	26,75	3,52	1,48	5,12	2,82	8,57	5,82	8,60
		median	360,40	79,45	11,99	8,32	30,06	10,34	69,39	23,53	35,51

The presented values are the average, standard deviation and median of, at least, three preparations

Abbreviations:

TFLi Inicial trifluralin
TFLf Final trifluralin
LIPi Initial lipid
LIPf Final lipid
(TFL/LIP)i Initial ratio TFL/LIP
(TFL/LIP)f Initial ratio TFL/LIP
LIPf/LIPi LIP recovery
TFLf/TFLi TFL recovery
I.E. Incorporation efficiency

TABLE 1b - Lipid composition effect on incorporation parameters of TFL in liposomes sized until 0.1 µm (cont.)

Formulation number	Lipidic composition Molar Ratio		TFLi (µg)	TFLf (µg)	LIPi (µmol)	LIPf (µmol)	(TFL/LIP)i (g/mol)	(TFL/LIP)f (g/mol)	LIPf/LIPi (%)	TFLf/TFLi (%)	I.E. (%)
11	DPPC:CHOL	average	318,22	58,03	9,11	6,29	34,72	8,85	69,09	16,84	24,93
	4:1	standard deviation	64,51	44,95	1,22	1,50	2,47	5,36	13,59	10,32	14,02
		median	310,97	48,90	9,18	6,07	33,86	9,97	76,57	15,72	29,44
12	PC:CHOL (Soya been)	average	397,89	109,66	10,45	5,80	39,69	18,89	56,55	27,45	49,54
	4:1	standard deviation	22,78	19,83	2,63	0,96	9,65	0,84	6,92	3,82	12,22
		median	395,90	119,49	11,93	6,02	35,10	18,53	55,12	29,09	52,78
13	DPPC:DPPG	average	305,04	33,09	8,71	3,83	35,03	8,83	44,42	10,90	25,40
	7:3	standard deviation	25,83	12,77	0,32	1,65	2,54	0,80	20,32	4,48	4,00
		median	293,19	35,20	8,85	4,30	34,43	8,59	48,59	10,52	24,95
14	DOPC:DOPG	average	323,50	109,74	10,35	5,48	31,31	19,61	52,13	33,51	63,77
	7:3	standard deviation	37,57	54,44	0,80	2,32	3,40	3,19	18,10	14,10	16,65
		median	324,80	100,53	10,37	4,55	32,35	20,71	43,88	35,24	64,03
15	PC:CHOL:PG	average	313,63	106,14	12,33	8,91	25,69	11,82	73,21	33,41	47,11
	10:5:1	standard deviation	44,63	45,41	2,19	1,21	3,48	4,64	11,70	11,11	20,89
		median	309,00	97,36	11,82	9,06	24,48	9,70	73,14	35,86	42,22
16	PC:CHOL:PI	average	337,98	111,78	12,23	7,86	27,98	13,36	62,87	31,73	48,78
	10:5:1	standard deviation	40,38	74,27	2,55	3,14	2,73	3,43	12,67	17,06	17,14
		median	320,85	71,02	11,13	6,54	27,76	11,95	62,82	22,13	43,05
17	PC:CHOL:SA	average	290,55	40,34	10,54	6,42	27,79	7,87	57,74	13,51	27,38
	10:5:1	standard deviation	35,45	26,53	1,89	3,96	2,09	6,49	26,86	7,79	20,76
		median	277,39	40,47	10,38	5,99	26,72	6,31	57,67	15,35	23,87
18	DPPC:CHOL:DPPG	average	324,80	14,83	9,03	4,39	35,87	6,58	48,86	4,36	17,71
	10:5:1	standard deviation	47,95	8,84	0,26	2,67	4,34	7,90	30,20	2,32	21,32
		median	338,63	19,39	9,15	5,69	37,01	3,41	61,73	5,32	8,62
19	PC:CHOL:DSPE-PEG	average	231,29	188,33	9,47	9,09	24,41	20,68	96,02	81,90	84,91
	3.7:1:0.3	standard deviation	13,16	34,07	0,06	1,48	1,26	0,46	16,20	17,32	5,97
		median	227,99	182,71	9,46	8,75	24,20	20,88	92,49	83,01	86,75
20	DSPC:CHOL:DSPE-PEG	average	322,82	159,37	10,20	7,22	32,73	25,65	69,44	50,96	76,60
	8:2:0.5	standard deviation	60,28	30,66	1,68	3,99	10,23	10,21	29,97	15,04	11,23
		median	350,48	163,74	10,45	5,73	34,86	30,25	68,21	53,53	78,47

The presented values are the average, standard deviation and median of, at least, three preparations

Abbreviations:

TFLi Inicial trifluralin *LIPi* Initial lipid *(TFL/LIP)i* Initial ratio TFL/LIP *LIPf/LIPi* LIP recovery

TFLf Final trifluralin *LIPf* Final lipid *(TFL/LIP)f* Initial ratio TFL/LIP *TFLf/TFLi* TFL recovery

I.E. Incorporation efficiency

From the analysis of **Table 1a** and **1b** important conclusion can be drawn, referring to the effect of the presence of cholesterol in lipid composition, the effect of electrically charged molecules in lipid composition and to the effect of lipids with different phase transition temperature. The obtained results evidence that TFL is better incorporated in liposomes with low content of cholesterol, as can be observed in **Figure 1** (in the formulation composed of PC:CHOL in a molar ratio of 4:1). The most significative difference was observed to the small size liposomes (VET100). In what concerns the presence of electrically charged molecules in the lipid composition it can be concluded that TFL is poorly incorporated in positively charged liposomes (PC:CHOL:SA) and that, in spite of the better results had been obtained with electrically neutral liposomes (PC:CHOL), negatively charged liposomes (PC:CHOL:PI and PC:CHOL:PG) present good values of incorporation, as can be seen in **Figure 2**. This is an important result, as it is known that negatively charged liposomes, after parenterical administration, have longer circulating times. In what concerns the use of lipids with different phase transition temperature, a comparison can be made between the results obtained for the liposomal formulations with PC, HPC and DSPC (increasing phase transition temperature) in different proportions with CHOL. For the formulations with lower proportion of cholesterol, the lipid with lower transition phase temperature (PC) revealed the best results for TFL incorporation. However, when the cholesterol proportion increases, the lipid with bigger transition phase temperature (DSPC) revealed the best results, even though the difference is not significative, as can be observed in **Figure 3**.

In vitro stability of two trifluralin liposomal formulations

The preparation of the liposomal formulations for this stability study *in vitro*, 4 mL initial volume containing 10 µmole/mL of lipid (PC:PG) in 4:1 molar ratio and 1 µmole/mL (335 µg/mL) of TFL, started by the addition of TFL to the lipid in chloroform, followed by evaporation of the solvent under nitrogen stream. The hydration of the resulting film was carried out by addition of 400 µL of 0,3 M

trehalose, stirring, 15 minute rest, addition of 400 µL more of 0,3 M tr halose, stirring again and resting again for more 15 minute and, finally, with the addition of 3200 µL of 0,3 M trehalose.

The so obtained liposomal formulations were sized by successive filtration under nitrogen pressure, through polycarbonate filters with pores of 5,0, 2,0, 1,0, 0,8, 0,6 and 0,4 µm, with two passages in this late filter (extrusion). The non-incorporated TFL, as it is insoluble in aqueous solutions, crystallises on a needle type structure and remains at the top of the filters. After extrusion through 0,4 µm filter, the formulations are split in two equal parts. With one of those parts extrusion procedure continues, now through diameter pore membranes of 0,2 and 0,1 µm, with two passages in the last filter. The two formulations obtained according to the previous process were kept at 4°C and samples for dosage of TFL were removed at days 0, 1, 2, 3, 4, 6, 8 and 10, being the result expressed by comparison with the value obtained for day O, in percentage. Immediately before the sampling, the formulations were microscopically observed for crystal detection that, if present, would be removed by centrifugation.

As can be seen in **Figure 4**, the two liposomal formulations (VET 400 and VET100) are stable, upon hydration, presenting 100% stability in the first 48 hours. The experience was repeated three times, being the presented values, the median of the results obtained for each time point.

Stability on storage of three different trifluralin liposomal formulations

The preparation of the liposomal formulations for this stability study *in vitro*, 45 mL initial volume containing 10 µmole/mL of lipid (DOPC:DOPG) in 7:3 molar ratio and 1 µmole/mL (335 µg/mL) of TFL, started by the addition of TFL to the lipid in chloroform, followed by evaporation of the solvent under nitrogen stream. The hydration of the resulting film was carried out by addition of 4,5 mL of 0,3 M trehalose, stirring, 30 minute rest, addition of 4,5 mL more of 0,3 M trehalose, stirring again and resting again for more 15 minute and, finally, with the addition of 36 mL of 0,3 M trehalose.

The so obtained liposomal formulations were sized by successive filtration under nitrogen pressure, through polycarbonate filters with pores of 5,0, 2,0, 1,0, 0,8, 0,6 and 0,4 µm, with two passages in this late filter (extrusion). The non-incorporated TFL, as it is insoluble in aqueous solutions, crystallises on a needle type structure and remains at the top of the filters. After extrusion through 0,4 µm filter, the formulations are split in two equal parts. With one of those parts extrusion procedure continues, now through diameter pore membranes of 0,2 and 0,1 µm, with two passages in the last filter. From the two formulations VET400 and VET 100 obtained according to the previous process, equal amounts were taken and mixed, being this mixture of the two previous formulations named as MIX liposomal formulation. The three liposomal formulations were then split by vials of 1 mL each, frozen at –70°C during 1 hour and lyophilised overnight. After the lyophilization procedure the vials were closed, under vacuum, sealed with aluminium caps and placed in the benchtop for the entire time of the stability study. For each experimental point (0, 0,03, 0,07, 0,13, 0,23, 0,47, 0,7, 1, 2, 3, 4, 5, 6 and 12 months), vials were opened, hydrated with deionised sterile water until the final volume of 1 mL. The vials containing the liposomal formulations were left to stand for 2 hours. The formulations were microscopically observed for crystal detection that, if present, would be removed by centrifugation. Quantification of TFL was carried out for each formulation and the results expressed as the percentage of TFL as compared to day 0 (final day of lyophilization).

As can be seen from Figure 5, the three liposomal formulations (VET 400, VET100 and MIX) are stable, in the lyophilised form, presenting, after one year of preparation followed by water hydration, TFL values bigger than 90% of the initial value. The experiment was conducted in triplicate and the presented values represent the median of the obtained values for each point.

Singl dos toxicity evaluation

This study was performed with a liposomal formulation of TFL with DOPC:DOPG 7:3 as the lipid composition and compared with a liposomal formulation with equal lipid composition without TFL (empty liposomes).

The preparation of the empty liposomal formulation for this single dose toxicity study, 400 mL initial volume containing 10 µmole/mL of lipid (DOPC:DOPG) in 7:3 molar ratio, started by measuring of lipid in chloroform, followed by evaporation of the solvent under nitrogen stream. The hydration of the resulting film was carried out by addition of 40 mL of 0,3 M sucrose, stirring, 30 minute rest, addition of 40 mL more of 0,3 M sucrose, stirring again and resting again for more 15 minute and, finally, with the addition of 320 mL of 0,3 M sucrose.

The so obtained empty liposomal formulation was sized by successive filtration under nitrogen pressure, through polycarbonate filters with pores of 5,0, 2,0, 1,0, 0,8, 0,6 and 0,4 µm, with two passages in this late filter (extrusion). After extrusion the liposomal formulation was submitted to ultracentrifugation at 49.000 rpm, for two hours, at 15°C. After ultracentrifugation, supernatant was removed and the pellet was ressuspended until 35 mL by addition of 0,3 M sucrose.

The preparation of the TFL liposomal formulation was performed according to the same process described for the empty formulation, with TFL being added to the initial solution of lipid in chloroform. After extrusion through 0,4 µm filter, the formulations are split in two equal parts. With one of those parts extrusion procedure continues, now through diameter pore membranes of 0,2 and 0,1 µm, with two passages in the last filter. The TFL formulations VET400 and VET100 obtained according to the previous process were mixed, being this mixture of the two previous formulations named as MIX liposomal formulation. The formulation was microscopically observed for crystal detection that, if present, would be removed by centrifugation.

The final lipid concentration was determined in both empty and TFL liposomal formulations being the late one adjusted in a way that both formulations

contained exactly the same concentration of lipid. After this adjustment the TFL concentration was determined in the TFL containing formulation.

The study was carried out in BALB/c male and female mice. The liposomal formulations were administered by two routes: intraperitoneal (i.p.) and intravenous (i.v.). The administered doses were 30, 20 and 10 mL/kg for the i.p. route and of 10, 5 and 2 mL/kg for the i.v. route of administration. 5 animal per group were used. The administered doses correspond to calculated doses of lipid (in both formulations) and of TFL (in the TFL containing formulation) presented in **Table 2**.

Table 2 – Single dose toxicity doses

Dose (mL/Kg)	Lipid (µmole/Kg)	TFL (mg/Kg)
30	2790	63.9
20	1860	42.6
10	930	21.3
5	465	10.7
2	186	4.3

All animals were weighted and the amount of liposomal formulation was calculated according to the measured weight in order to achieve the desired dose, in mL/kg. One animal group per sex was injected with 0,3 M sucrose as control group

The animals were observed at regular intervals, during 48 hours after administration, for detection of behaviour changes. After that period, animals were euthanised, weighted and, from each animal, heart, spleen, liver and kidneys were removed, weighted and observed for macroscopical changes. Relative organ weight was calculated as the ratio between the organ weight and the weight of the animal. The obtained results are presented from **Table 3** to **20**.

Tabl 3 – Absolute animal weight (empty liposomes)

Dose (mL/Kg)		*Administration routes*			
		i.v.		i.p.	
		Males	Females	Males	Females
Control	average	3,91E+01	2,93E+01	3,69E+01	2,78E+01
	standard deviation	3,67E+00	1,61E+00	1,94E+00	1,02E+00
2	average	3,75E+01	2,92E+01		
	standard deviation	1,42E+00	1,99E+00		
5	average	3,80E+01	2,76E+01		
	standard deviation	2,73E+00	8,12E-01		
10	average	3,67E+01	2,92E+01	3,75E+01	2,73E+01
	standard deviation	9,15E-01	1,73E+00	1,45E+00	1,33E+00
20	average			3,55E+01	2,92E+01
	standard deviation			4,60E+00	2,91E+00
30	average			3,48E+01	2,94E+01
	standard deviation			1,52E+00	1,60E+00

Table 4 – Absolute heart weight (empty liposome)

Dose (mL/Kg)		*Administration routes*			
		i.v.		i.p.	
		Males	Females	Males	Females
Control	average	1,70E-01	1,42E-01	2,07E-01	1,44E-01
	standard deviation	2,55E-02	5,20E-03	2,38E-02	2,13E-02
2	average	1,87E-01	1,55E-01		
	standard deviation	8,81E-03	1,67E-02		
5	average	1,85E-01	1,37E-01		
	standard deviation	1,95E-02	2,09E-02		
10	average	1,96E-01	1,50E-01	2,11E-01	1,33E-01
	standard deviation	3,68E-02	6,17E-03	3,63E-02	2,15E-02
20	average			2,15E-01	1,34E-01
	standard deviation			3,04E-02	1,07E-02
30	average			1,92E-01	1,49E-01
	standard deviation			1,18E-02	2,47E-02

Tabl 5 – Absolute liver weight (empty liposomes)

Dose (mL/Kg)		*Administration routes*			
		i.v.		i.p.	
		Males	Females	Males	Females
Control	average	1,72E+00	1,31E+00	1,89E+00	1,07E+00
	standard deviation	2,08E-01	4,25E-02	2,07E-01	9,86E-02
2	average	1,72E+00	1,24E+00		
	standard deviation	7,32E-02	1,39E-01		
5	average	1,62E+00	1,12E+00		
	standard deviation	1,74E-01	8,91E-02		
10	average	1,68E+00	1,21E+00	1,96E+00	1,06E+00
	standard deviation	8,40E-02	1,40E-01	1,14E-01	7,88E-02
20	average			1,95E+00	1,20E+00
	standard deviation			1,12E-01	1,68E-01
30	average			1,58E+00	1,19E+00
	standard deviation			1,25E-01	1,40E-01

Table 6 – Absolute spleen weight (empty liposomes)

Dose (mL/Kg)		*Administration routes*			
		i.v.		i.p.	
		Males	Females	Males	Females
Control	average	1,03E-01	9,46E-02	1,26E-01	1,04E-01
	standard deviation	1,08E-02	5,36E-02	2,51E-02	2,66E-02
2	average	1,10E-01	9,86E-02		
	standard deviation	1,51E-02	4,86E-02		
5	average	1,21E-01	7,60E-02		
	standard deviation	9,11E-03	2,53E-02		
10	average	1,16E-01	1,07E-01	1,06E-01	7,46E-02
	standard deviation	1,14E-02	2,55E-02	8,21E-03	2,36E-02
20	average			1,27E-01	1,01E-01
	standard deviation			1,88E-02	1,88E-02
30	average			1,08E-01	8,97E-02
	standard deviation			8,35E-03	1,86E-02

Tabl 7 – Absolute kidneys w ight (empty liposomes)

Dose (mL/Kg)		*Administration routes*			
		i.v.		i.p.	
		Males	Females	Males	Females
Control	average	6,37E-01	3,64E-01	6,22E-01	3,11E-01
	standard deviation	7,83E-02	2,62E-02	5,57E-02	4,00E-02
2	average	6,13E-01	3,63E-01		
	standard deviation	6,42E-02	2,76E-02		
5	average	6,46E-01	3,20E-01		
	standard deviation	1,11E-01	4,23E-02		
10	average	6,63E-01	3,99E-01	6,02E-01	2,95E-01
	standard deviation	6,17E-02	4,82E-02	6,61E-02	2,20E-02
20	average			5,24E-01	3,00E-01
	standard deviation			2,62E-01	1,80E-02
30	average			6,29E-01	3,17E-01
	standard deviation			6,37E-02	2,64E-02

Table 8 – Absolute animal weight (TFL containing liposomes)

Dose (mL/Kg)		*Administration routes*			
		i.v.		i.p.	
		Males	Females	Males	Females
Control	average	2,61E+01	2,06E+01	2,64E+01	1,96E+01
	standard deviation	1,42E+00	1,13E+00	1,82E+00	1,93E+00
2	average	2,52E+01	1,89E+01		
	standard deviation	7,53E-01	1,28E+00		
5	average	2,58E+01	1,97E+01		
	standard deviation	1,99E+00	7,31E-01		
10	average	2,62E+01	2,01E+01	2,45E+01	2,05E+01
	standard deviation	1,32E+00	9,32E-01	1,79E+00	1,60E+00
20	average			2,71E+01	2,07E+01
	standard deviation			2,32E+00	6,96E-01
30	average			1,96E+01	2,02E+01
	standard deviation			1,93E+00	2,67E+00

Table 9 – Absolute heart weight (TFL containing liposomes)

Dose (mL/Kg)		*Administration routes*			
		i.v.		i.p.	
		Males	Females	Males	Females
Control	average	1,43E-01	1,08E-01	1,38E-01	9,60E-02
	standard deviation	1,28E-02	1,10E-02	1,92E-02	8,98E-03
2	average	1,39E-01	1,05E-01		
	standard deviation	9,49E-03	1,18E-02		
5	average	1,37E-01	1,14E-01		
	standard deviation	7,03E-03	8,61E-03		
10	average	1,54E-01	1,04E-01	1,30E-01	9,65E-02
	standard deviation	9,80E-03	1,48E-02	1,56E-02	9,46E-03
20	average			1,57E-01	1,05E-01
	standard deviation			3,50E-02	5,74E-03
30	average			1,34E-01	9,62E-02
	standard deviation			1,55E-02	1,17E-02

Table 10 – Absolute liver weight (TFL containing liposomes)

Dose (mL/Kg)		*Administration routes*			
		i.v.		i.p.	
		Males	Females	Males	Females
Control	average	1,32E+00	9,71E-01	1,19E+00	8,80E-01
	standard deviation	5,16E-02	8,62E-02	7,82E-02	1,10E-01
2	average	1,20E+00	9,63E-01		
	standard deviation	1,21E-01	8,88E-02		
5	average	1,31E+00	9,79E-01		
	standard deviation	1,76E-01	2,86E-02		
10	average	1,17E+00	1,00E+00	1,21E+00	1,01E+00
	standard deviation	6,32E-01	5,03E-02	1,40E-01	8,45E-02
20	average			1,40E+00	9,97E-01
	standard deviation			1,30E-01	6,59E-02
30	average			1,27E+00	9,19E-01
	standard deviation			1,48E-01	1,73E-01

Table 11 – Absolute spleen weight (TFL containing liposomes)

Dose (mL/Kg)		*Administration routes*			
		i.v.		i.p.	
		Males	Females	Males	Females
Control	average	9,02E-02	8,52E-02	8,25E-02	7,66E-02
	standard deviation	5,55E-03	8,24E-03	3,97E-03	1,75E-02
2	average	8,65E-02	7,98E-02		
	standard deviation	1,28E-02	8,21E-03		
5	average	9,47E-02	9,41E-02		
	standard deviation	2,51E-02	4,85E-03		
10	average	1,02E-01	9,16E-02	9,52E-02	9,05E-02
	standard deviation	2,24E-02	6,22E-03	7,56E-03	8,78E-03
20	average			9,45E-02	9,65E-02
	standard deviation			8,13E-03	5,22E-03
30	average			1,10E-01	8,72E-02
	standard deviation			4,03E-02	1,58E-02

Table 12 – Absolute kidneys weight (TFL containing liposomes)

Dose (mL/Kg)		*Administration routes*			
		i.v.		i.p.	
		Males	Females	Males	Females
Control	average	3,86E-01	2,32E-01	3,56E-01	2,21E-01
	standard deviation	3,53E-02	2,41E-02	2,80E-02	3,16E-02
2	average	3,52E-01	2,13E-01		
	standard deviation	1,30E-02	1,98E-02		
5	average	3,62E-01	2,15E-01		
	standard deviation	4,37E-02	4,90E-02		
10	average	3,90E-01	2,25E-01	3,36E-01	2,17E-01
	standard deviation	2,35E-02	1,92E-02	3,23E-02	2,46E-02
20	average			3,61E-01	2,38E-01
	standard deviation			4,23E-02	1,88E-02
30	average			3,48E-01	2,14E-01
	standard deviation			2,94E-02	1,76E-02

Tabl 13 – Relative heart weight (empty liposomes)

Dose (mL/Kg)		*Administration routes* i.v. Males	i.v. Females	i.p. Males	i.p. Females
Control	average	4,35E-03	4,88E-03	5,61E-03	5,18E-03
	standard deviation	4,87E-04	3,60E-04	7,34E-04	7,66E-04
2	average	4,99E-03	5,31E-03		
	standard deviation	1,62E-04	2,77E-04		
5	average	4,88E-03	4,96E-03		
	standard deviation	5,63E-04	8,06E-04		
10	average	4,88E-03	5,16E-03	5,61E-03	4,91E-03
	standard deviation	3,60E-04	3,89E-04	9,03E-04	8,90E-04
20	average			6,08E-03	4,61E-03
	standard deviation			8,16E-04	4,65E-04
30	average			5,52E-03	5,07E-03
	standard deviation			2,82E-04	8,75E-04

Table 14 – Relative liver weight (empty liposomes)

Dose (mL/Kg)		*Administration routes* i.v. Males	i.v. Females	i.p. Males	i.p. Females
Control	average	4,39E-02	4,48E-02	5,12E-02	3,87E-02
	standard deviation	1,45E-03	2,38E-03	4,75E-03	3,30E-03
2	average	4,58E-02	4,27E-02		
	standard deviation	2,02E-03	2,84E-03		
5	average	4,27E-02	4,07E-02		
	standard deviation	3,10E-03	2,43E-03		
10	average	4,58E-02	4,14E-02	5,24E-02	3,86E-02
	standard deviation	2,98E-03	3,03E-03	3,36E-03	1,43E-03
20	average			5,59E-02	4,09E-02
	standard deviation			9,62E-03	3,32E-03
30	average			4,53E-02	4,04E-02
	standard deviation			2,71E-03	4,22E-03

Table 15 – Relative spleen weight (empty liposomes)

Dose (mL/Kg)		*Administration routes*			
		i.v.		i.p.	
		Males	Females	Males	Females
Control	average	2,65E-03	3,29E-03	3,42E-03	3,75E-03
	standard deviation	3,51E-04	1,95E-03	6,65E-04	9,22E-04
2	average	2,94E-03	3,33E-03		
	standard deviation	4,13E-04	1,59E-03		
5	average	3,19E-03	2,75E-03		
	standard deviation	2,49E-05	9,28E-04		
10	average	3,18E-03	3,67E-03	2,81E-03	2,74E-03
	standard deviation	3,57E-04	7,30E-04	1,26E-04	8,96E-04
20	average			3,66E-03	3,46E-03
	standard deviation			9,83E-04	4,51E-04
30	average			3,11E-03	3,05E-03
	standard deviation			1,42E-04	6,26E-04

Table 16 – Relative kidneys weight (empty liposomes)

Dose (mL/Kg)		*Administration routes*			
		i.v.		i.p.	
		Males	Females	Males	Females
Control	average	1,63E-02	1,24E-02	1,69E-02	1,12E-02
	standard deviation	1,36E-03	4,49E-04	1,81E-03	1,12E-03
2	average	1,63E-02	1,25E-02		
	standard deviation	1,27E-03	3,47E-04		
5	average	1,69E-02	1,16E-02		
	standard deviation	2,22E-03	1,64E-03		
10	average	1,81E-02	1,37E-02	1,60E-02	1,08E-02
	standard deviation	1,79E-03	1,53E-03	1,50E-03	1,16E-03
20	average			1,52E-02	1,03E-02
	standard deviation			8,41E-03	6,26E-04
30	average			1,81E-02	1,08E-02
	standard deviation			1,51E-03	1,10E-03

Table 17 – Relative heart weight (TFL containing liposomes)

Dose (mL/Kg)		*Administration routes*			
		i.v.		i.p.	
		Males	Females	Males	Females
Control	average	5,49E-03	5,27E-03	5,25E-03	4,90E-03
	standard deviation	4,52E-04	5,20E-04	7,39E-04	1,97E-04
2	average	5,50E-03	5,56E-03		
	standard deviation	4,97E-04	7,85E-04		
5	average	5,35E-03	5,79E-03		
	standard deviation	5,73E-04	5,42E-04		
10	average	5,90E-03	5,20E-03	5,28E-03	4,70E-03
	standard deviation	5,87E-04	7,05E-04	3,02E-04	2,23E-04
20	average			5,77E-03	5,07E-03
	standard deviation			1,04E-03	2,44E-04
30	average			5,19E-03	4,79E-03
	standard deviation			4,46E-04	3,87E-04

Table 18 – Relative liver weight (TFL containing liposomes)

Dose (mL/Kg)		*Administration routes*			
		i.v.		i.p.	
		Males	Females	Males	Females
Control	average	5,07E-02	4,72E-02	4,50E-02	4,48E-02
	standard deviation	3,17E-03	3,28E-03	2,38E-03	1,82E-03
2	average	4,77E-02	5,09E-02		
	standard deviation	4,03E-03	3,25E-03		
5	average	5,06E-02	4,98E-02		
	standard deviation	4,96E-03	1,78E-03		
10	average	4,43E-02	4,99E-02	4,93E-02	4,93E-02
	standard deviation	2,34E-02	2,75E-03	4,63E-03	1,43E-03
20	average			5,15E-02	4,81E-02
	standard deviation			1,31E-03	1,96E-03
30	average			4,93E-02	4,53E-02
	standard deviation			3,60E-03	2,70E-03

Tabl 19 – Relative spleen weight (TFL containing liposomes)

Dose (mL/Kg)		*Administration routes*			
		i.v.		i.p.	
		Males	Females	Males	Females
Control	average	3,47E-03	4,15E-03	3,13E-03	3,87E-03
	standard deviation	2,14E-04	3,94E-04	1,04E-04	6,85E-04
2	average	3,42E-03	4,22E-03		
	standard deviation	4,35E-04	3,83E-04		
5	average	3,65E-03	4,78E-03		
	standard deviation	8,45E-04	1,91E-04		
10	average	3,86E-03	4,57E-03	3,90E-03	4,41E-03
	standard deviation	6,87E-04	2,34E-04	4,63E-04	1,18E-04
20	average			3,49E-03	4,66E-03
	standard deviation			2,25E-04	2,21E-04
30	average			4,24E-03	4,32E-03
	standard deviation			1,33E-03	4,76E-04

Table 20 – Relative kidneys weight (TFL containing liposomes)

Dose (mL/Kg)		*Administration routes*			
		i.v.		i.p.	
		Males	Females	Males	Females
Control	average	1,48E-02	1,13E-02	1,35E-02	1,12E-02
	standard deviation	1,28E-03	5,42E-04	1,03E-03	5,59E-04
2	average	1,40E-02	1,12E-02		
	standard deviation	8,36E-04	5,44E-04		
5	average	1,40E-02	1,09E-02		
	standard deviation	1,19E-03	2,57E-03		
10	average	1,49E-02	1,12E-02	1,37E-02	1,06E-02
	standard deviation	6,29E-04	6,70E-04	6,60E-04	7,15E-04
20	average			1,33E-02	1,15E-02
	standard deviation			8,20E-04	5,48E-04
30	average			1,35E-02	1,07E-02
	standard deviation			8,32E-04	7,50E-04

The obtained values were statistically treated for the significance of variations (p=0,005). Obtained results for absolute weights (full animal and separate organs) in both formulations were compared between themselves and with absolute weight of control group. The total absence of toxicity of any of the injected formulations was concluded from the statistical analysis.

Biological activity evaluation

In order to evaluate the biological activity of TFL liposomal formulations prepared according to the present invention, one animal model of leishmaniasis was selected. BALB/c mice were infected with 2 x 10^7 (i.v.) LV-9 (*Leishmania donovani*) parasites, obtained from the London School of Hygiene and Tropical Medicine. The groups (5 animals per group) and treatment schedules are presented in **Table 21**.

Table 21 – Biological activity

Formulation lipidic composition	Dose (mg TFL/kg)	N^{er} of Doses	Inhibition %
DOPC:DOPG 7:3	15	5	62
		3	17
		1	80
DSPC:CHOL 4:1	15	5	53
		3	92
		1	91
PC:PG 4:1	15	5	47
		3	88
		1	60
PC:CHOL:PI 3.7:1:0,3	5	5	57
		3	52
		1	68
PC:CHOL:DSPE-PEG (2000) 3.7:1:0,3	4	5	75
		3	74
DOPC:DOPG 7:3 (dialysed)	0,6	5	86

Treatments started 7 days post-infection and animals were euthanised 15 days post-infection. Liver was removed and weighted from each animal and amastigote counting was performed in each one by smear impression. The infection was calculated through an appropriate mathematical equation. The so obtained results are expressed in **Table 21**.

The first conclusion is that, due to the liposomal incorporation of TFL, parenteric administration of TFL is possible.

All TFL liposomal formulations were able to reduce infection in this model.

Claims

1. A liposomal formulation characterized by the fact of containing one dinitroaniline incorporated or encapsulated.

2. A liposomal formulation, according to claim 1, characterized by the fact of the dinitroaniline being trifluralin.

3. A liposomal formulation, according to any of the claims 1 and 2, characterized by the fact of containing liposomes with diameter varying from 0.01 µm to 50 µm.

4. A liposomal formulation, according to any of the claims 1 to 3, characterized by the fact of mixing populations of particles with different diameter.

5. A liposomal formulation, according to any of the claims 1 to 4, characterized by the fact of mixing populations of particles, respectively bigger and lower than 100 nm.

6. Liposomal formulations, according to any of the previous claims, characterized by the fact of being prepared with any of the following lipids, hydrogenated or not, individually or in mixtures, in any molar ratio: distearoylphosphatidylcholine (DSPC), phosphatidylcholine (PC), cholesterol (Chol) or derivatives, sphingomielin (SM), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), phosphatidylglycerol (PG), dimiristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), gangliosides, ceramides, phosphatidylinositol (PI), phosphatydic acid (PA), dicetylphosphate (DcP), dimiristoylphosphatidylglycerol, (DMPG), stearylamine (SA), dipalmitoylphosphatidylglycerol (DPPG) and other synthethic lipids.

7. Process for the preparation of a liposomal formulation containing one dinitroaniline, characterized by:

- obtention of a liposomal preparation containing a dinitroaniline by hydration of a lipidic film containing the dinitroaniline
- lyophilization of the dinitroaniline liposomal formulation
- rehydration of the dehydrated liposomal formulation

8. Process according to claim 7, characterized by the performing of a sizing step of the dinitroaniline liposomal formulation in order to reduce the vesicle diameter, done previously to the dehydration step.

9. Process, according to claim 8, characterized by the performing of the sizing step by extrusion under pressure through porous membranes.

10. Process, according to any of the claims 7 to 9, characterized by the fact that the hydration is carried out by the addition of a small amount of an aqueous solution, followed by the addition of the remaining volume of the aqueous solution, after a resting period.

11. Process, according to claim 10, characterized by the fact of using, in the hydration steps, a non-saline solution.

12. Process, according to claim 11, characterized by the fact of performing the rehydration steps with saccharose, trehalose, glucose or any other sugar solution.

13. Process, according to any of the claims 7 to 12, characterized by the fact of mixing different diameter particle populations.

14. Process, according to claim 13, characterized by the fact of mixing particles that, after sizing, present population with diameters of, respectively, bigger and lower than 100 nm.

15. Process, according to claim 14, characterized by the fact of performing the sizing step according to claim 9.

16. Process, according to any of the claims 7 to 9 or 13 to 15, characterized by the fact that the hydration is performed according to claim 10.

17. Process, according to any of the claims 7 to 10 or 13 to 16, characterized by the fact of using in the hydration step a solution according to claim 11.

18. Process, according to any of the claims 7 to 11 or 13 to 17, characterized by the fact of using solutions according to claim 12.

19. Process, according to any of the claims 7 to 18, characterized by the use of any of the following lipids, hydrogenated or not, individually or in mixtures, in any molar ratio: distearoylphosphatidylcholine (DSPC), phosphatidylcholine (PC), cholesterol (Chol) or derivatives, sphingomielin (SM), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), phosphatidylglycerol (PG), dimiristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), gangliosides, ceramides, phosphatidylinositol (PI), phosphatydic acid (PA), dicetylphosphate (DcP), dimiristoylphosphatidylglycerol, (DMPG), stearylamine (SA), dipalmitoylphosphatidylglycerol (DPPG) and other synthethic lipids.

20. Process, according to any of the claims 7 to 19, characterized by the fact of the dinitroaniline is trifluralin.

21. Process, according to any of the claims 1 to 6, when prepared by a process according to any of the claims 7 to 20.

22. Use of the liposomal formulations for the treatment in humans or animals, characterized by the use of a therapeutic efficient quantity of a dinitroaniline liposomal formulation according to any of the claims 1 to 6 and 21.

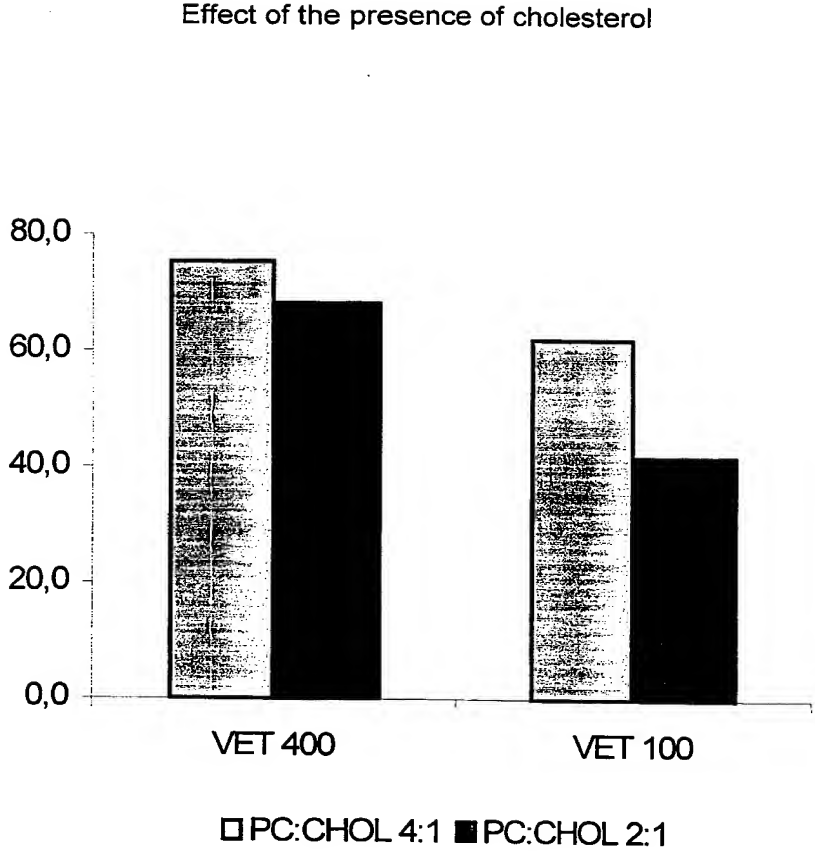
Effect of the presence of cholesterol
80,0
60,0
40,0
20,0
0,0
VET 400
VET 100
PC:CHOL 4:1
PC:CHOL 2:1

Figure 1

Effect of the presence of electric charge in the lipid membrane

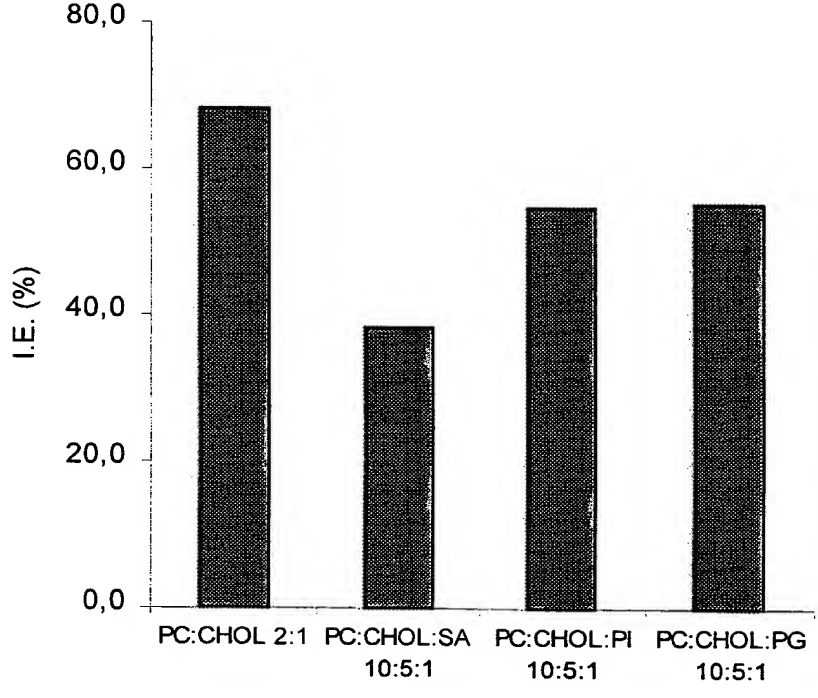

Figure 2

Effect of transition phase temperature of lipids

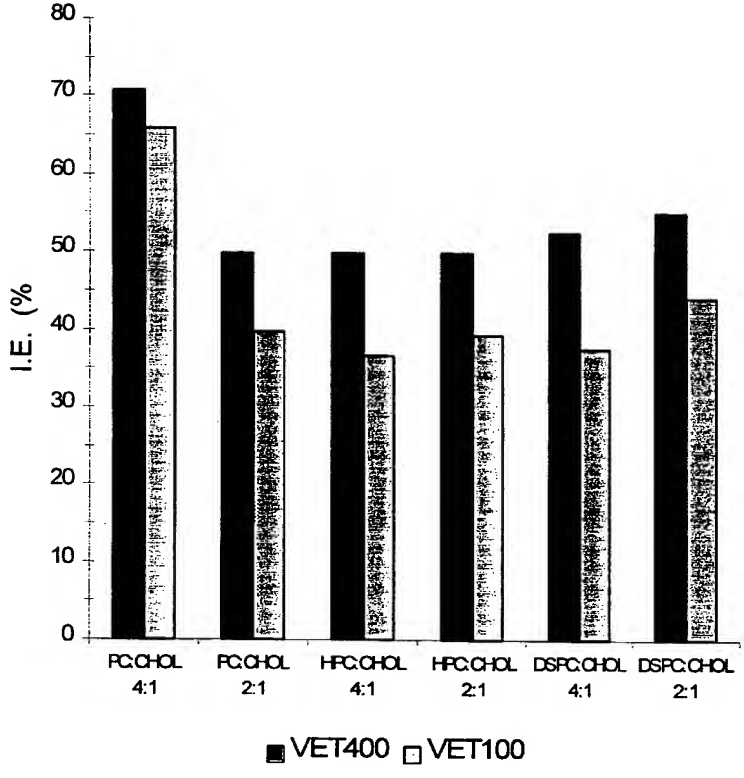

Figure 3

In vitro stability of two formulations of PC:PG 4:1

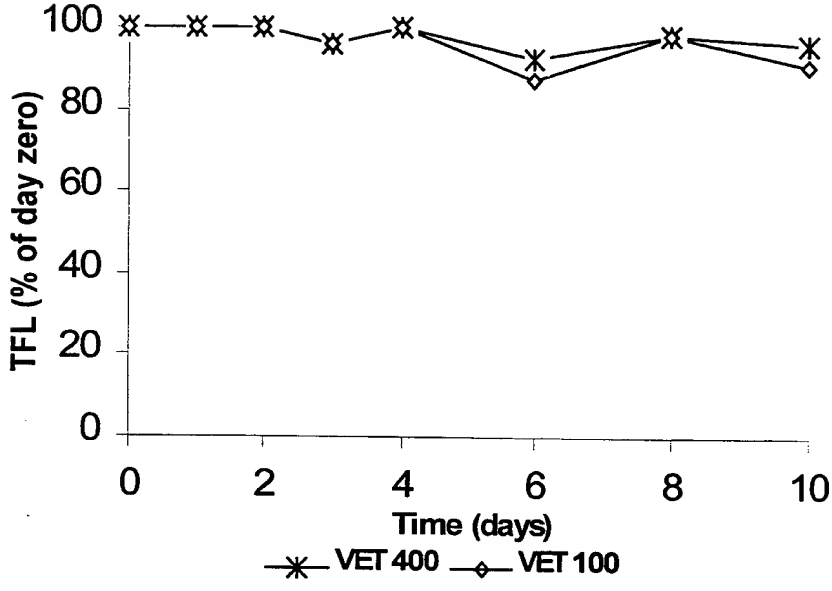

Figure 4

Stability on storage of three liposomal formulations of DOPC:DOPG 7:3

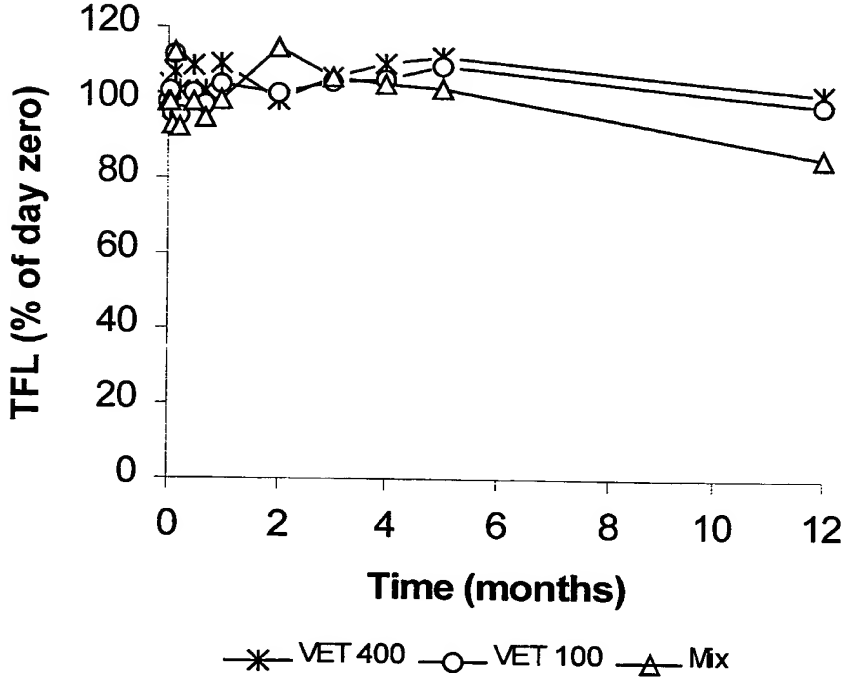

Figure 5